Cell cycle: **Cull and destroy**

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A newly discovered family of proteins homologous to yeast Cdc53, called cullins, may play a key role in the targeting of cell-cycle regulators, such as cyclins, for destruction by ubiquitin-dependent proteolysis.

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The covalent addition of ubiquitin to proteins directs their degradation by the proteasome [1]. Ubiquitination enzymes promote the destruction of misfolded, damaged or constitutively unstable proteins. Furthermore, the ubiquitination of specific targets has been shown to be critical for controlling cell-cycle progression and sensing cellular damage. The diversity of potential ubiquitination targets and the large family of ubiquitination enzymes raises the question of how specific proteins are targeted for ubiquitination. Recent studies have suggested that the yeast Cdc53 protein specifically associates with the phosphorylated form of a cyclin that is ubiquitinated at a specific stage of the cell cycle [2]. A family of proteins, called 'cullins', that are homologous to Cdc53 has been discovered. Cullins may bind to distinct target proteins, providing one explanation for how the ubiquitination system discriminates among its substrates and how phosphorylation may be used as a regulatory signal for initiating protein destruction.

The ubiquitin-dependent proteolysis machinery

Protein degradation by the ubiquitin pathway requires distinct enzymatic steps (Fig. 1). In the first step, a ubiquitin-activating enzyme (E1) uses ATP to form a thiolester bond between itself and ubiquitin. This activated ubiquitin is then transferred to one of a family of ubiquitin-conjugating enzymes (E2s). Some E2s directly catalyze formation of an isopeptide bond between the carboxyl terminus of ubiquitin and the ϵ-amino group of an internal lysine residue in a substrate protein; others require a ubiquitin ligase (E3) to transfer ubiquitin from the E2 to the substrate. The E2s provide substantial substrate specificity to the ubiquitination reaction [3]. It is not clear whether E3s form a single family of enzymes, nor do we understand their biochemical action in all cases. Some E3s form a ubiquitin–thiolester linkage; others may directly couple E2s to the substrate molecule. The latter role has been suggested for the 'anaphase promoting complex' (APC), a multiprotein complex critical for the destruction of cyclin B in mitosis [4,5].

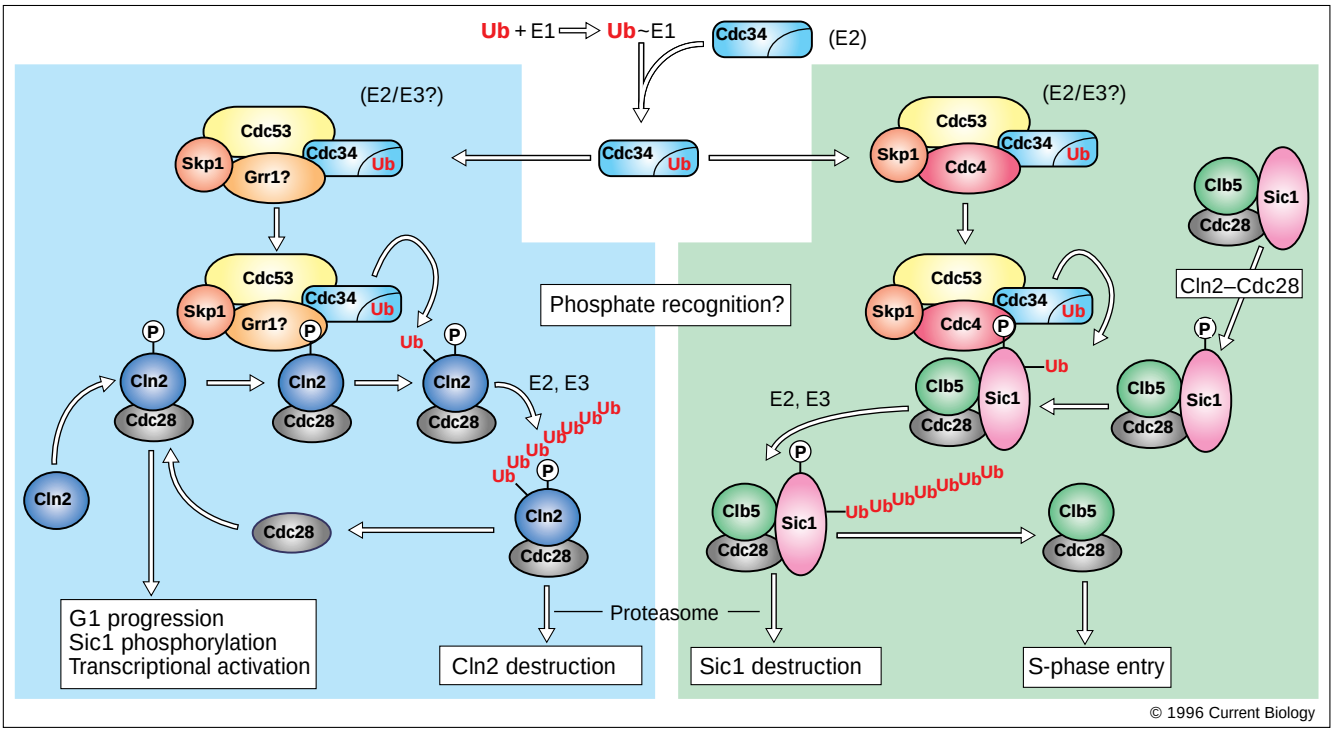
Within a given protein substrate, a small number of lysines are typically used for ubiquitination. Monoubiquitination is not sufficient, however, to target a protein for destruction — long polyubiquitin chains must be formed to direct substrates to the proteasome for degradation *in vivo*. Polyubiquitination may involve distinct E2s or E2/E3 combinations and more than one kind of ubiquitin–ubiquitin linkage in any chain [1]. The dynamics and regulation of chain formation are not well understood. Opposing the action of the ubiquitinating enzymes are a large group of de-ubiquitinating enzymes or isopeptidases [1]. Deubiquitination may ensure fidelity or cooperativity in the ubiquitination process, thereby avoiding spurious protein destruction. De-ubiquitinating enzymes may also be important for recycling ubiquitin after proteasome-mediated destruction of a specific protein [1].

Signal sequences and regulation by phosphorylation

The discovery that cyclin B undergoes rapid, ubiquitin-dependent destruction in mitosis explained why cyclin B oscillates in abundance every cell-cycle [6]. A cyclin B sequence required for its mitotic destruction, the 'destruction box', was identified and turns up in other mitotically destroyed proteins. Degradation of cyclin B *via* the destruction box appears to be regulated at the level of the mitotic destruction machinery: the APC appears to be activated in mitosis [4,5,7], presumably by phosphorylation, although the detailed mechanism is not known.

Another important type of degradation signal is the 'PEST' sequence, the acronym being the single-letter code for the amino acids abundant in such sequences. PEST sequences are found in unstable proteins, including the yeast cyclins Cln2 and Cln3 [3]. Although mutations in PEST sequences show that they confer instability, they are neither essential nor sufficient for targeting proteins for destruction [8]. Phosphorylation is a critical modification for degradation of the PEST-containing cyclin Cln2. Phosphorylation of Cln2 occurs upon binding to its associated Cdc28 protein kinase (the budding yeast Cdc2 homologue) and requires Cdc28 kinase activity [9]. Mutagenesis experiments showed that phosphorylation of Cln2, presumably by Cdc28, is required for its instability, providing a self-limiting activation of Cdc28 by Cln2. Although a highly stabilized, mutant form of Cln2 that lacks the phosphorylation sites can rescue viability, it accelerates progression though G1 and makes yeast resistant to growth arrest in response to nutrient starvation or pheromone. Cyclin instability thus appears to be critical for setting the rate of G1 progression in response to extracellular cues.

Figure 1



Multiple Cdc34/Cdc53 complexes regulate destruction of cyclins and Cdk inhibitors. See text for details.

Control of G1 progression by Cdc34, Cdc4 and Cdc53

Ubiquitin-dependent proteolysis was shown to have an important physiological role when a protein critical for G1 progression, Cdc34, was found to be homologous to E2s [10]. Genetic analysis showed that Cdc34, Cdc4 and Cdc53 are critical for a step in G1 progression after 'START' — the key point when a budding yeast commits to cell division — but before the initiation of DNA replication. Genetic interactions further suggested that these three proteins might function in a common pathway and physically interact [3]. It has recently been confirmed that Cdc53 can bind both Cdc34 and Cdc4, and one or several Cdc53-containing complexes may exist in the cell [2].

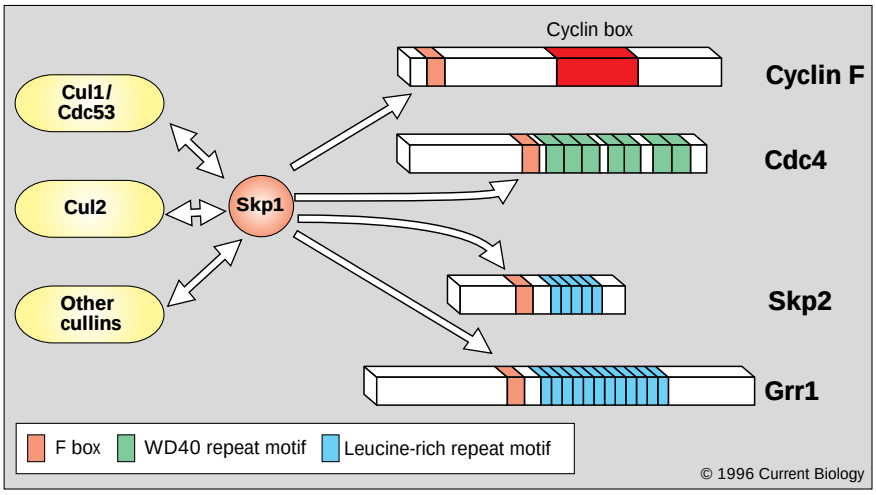
A central role for Cdc34 in G1 was recently demonstrated by Nasmyth and coworkers [11], who showed that it is required for the degradation of Sic1, an inhibitor of cyclin-dependent kinases (Cdks). Although not required for viability, Sic1 accumulates late in mitosis and throughout G1, and is degraded after START. Sic1 restrains S-phase entry by binding to the cyclin–Cdk complex Clb5–Cdc28, which accelerates passage through S phase. In *cdc34*^{ts}, *cdc4*^{ts} or *cdc53*^{ts} mutants, Sic1 accumulates and cells fail to enter S phase (Fig. 1). Dramatically, deletion of *SIC1* removed the block to S-phase entry in *cdc34*^{ts}, *cdc4*^{ts} or *cdc53*^{ts} mutants, so Sic1 appears to be the critical target for the Cdc34 pathway in controlling the G1–S transition. The *SIC1* deletion did not, however, rescue the ability of these mutant strains to divide, suggesting that there must be other critical substrates for the Cdc34 pathway.

That yeast cyclins Cln1, Cln2 and Cln3 are required for G1 progression suggested that their stability may determine the rate of G1 progression. This view is supported by biochemical and genetic evidence that the Cdc34 pathway is required for Cln destruction *in vitro* and *in vivo* [8,12], and by the finding that yeast expressing a stabilized Cln fail to respond to nutritional or pheromone arrest [9]. In addition to Cdc53 and Cdc34, a protein with homology to Cdc4, called Grr1, may be important for determining Cln2 stability [13].

Cdc34 also appears to be required for instability of the transcription factor Gcn4, which induces transcription of genes required for amino-acid biosynthesis [14]. Under nutritional starvation, Gcn4 is stabilized, promoting transcription of genes encoding amino-acid biosynthesis enzymes. When yeast cells are provided with amino acids in rich media, Gcn4 is targeted for ubiquitination *via* its PEST sequences. In *cdc34* mutants, Gcn4 is stabilized, suggesting that Cdc34 is required for Gcn4 instability. It is possible that the Cdc34 pathway may itself be modulated by nutritional status, possibly mediating a checkpoint control [3]. The human homologue of Cdc34 is capable of

Figure 2

Skp1 associates with multiple F-box-containing proteins and cullins to produce diverse complexes. Several of the F-box-containing proteins themselves contain sequences thought to mediate protein–protein interactions, including WD-40 and leucine-rich repeats and the cyclin box (see [17] for additional examples).



causing ubiquitination of p27, an inhibitor of the cyclin E–Cdk2 kinase [15]; cyclin E is required for S-phase entry and its inhibitor p27 appears to be activated by transforming growth factor-β (TGF-β). Cdc34 may thus mediate growth factor signaling in multicellular eukaryotes.

A solution to the recognition problem for ubiquitination substrates was recently suggested by Tyers and colleagues [2], who observed that Cdc53 binds specifically to phosphorylated Cln2, but not to unphosphorylated Cln2 or B-type cyclins. Clns are unstable when phosphorylated, and measurements of Cln stability in *cdc53* or *cdc34* mutant strains confirmed that Cdc53 is required for Cln instability. Tyers' group [2] additionally showed that Cdc53 is itself ubiquitinated and interacts directly with Cdc34. As Cdc53 interacts with both the substrate protein (Cln) and with the apparent E2 (Cdc34), they argue that Cdc53 is part of an E3. Although Cdc53 may direct the specific interaction of phosphorylated species, the interaction between Cdc53 and phosphorylated substrate was not shown to be direct. Other proteins, including Cdc4 or Grr1, may provide a bridge between Cdc53, Cdc34 and phosphorylated substrate (Fig. 1).

Skp1, Sic1 destruction and the F box

Two novel 'S-phase kinase-associated' proteins, Skp1 and Skp2, were recently discovered in association with cyclin A–Cdk2 complexes [16]. Anti-Skp2 antibodies blocked S-phase entry, suggesting that Skp2 has a direct role in DNA replication control. Genetic experiments pointed to a direct interaction between Skp1 and Cdc4, explained by the discovery that Skp1 binds to a structural motif within Cdc4, called the F box (Fig. 2) [17]. F boxes are found in many proteins, including cyclin F and Skp2, explaining Skp1's association with these proteins. Skp1 was found to be required for the G1–S and G2–M transitions [17]. Sic1 and Cln2 are stabilized in *skp1* mutants, suggesting that Skp1 may act, in concert with Cdc34, Cdc4 and Cdc53, to promote the degradation of specific substrates required for the G1–S (Fig. 1) and G2–M transitions.

A different possible function for Skp1 was suggested by the finding that Skp1 associates with the yeast kinetochore complex, CBF3 [18]. CBF3 is a complex of three proteins, p110, p64 and p58, which bind to a defined site, *CDEIII*, in centromere DNA. Mutations in these kinetochore components cause chromosome missegregation and loss of viability. To find gene products that interact with CBF3, Hieter and coworkers [18] screened for high-copy suppressors of mutations in the p58 gene, *CTF13*. The suppressors occurred in either *CTF13* itself or in *SKP1*. Skp1 was subsequently shown to be part of the CBF3–*CDEIII* complex, and *skp1* mutants were found to exhibit chromosome missegregation and cell-cycle arrest with a short mitotic spindle, indicating a pre-anaphase block. Reconstitution of the CBF3 complex also demonstrated that Skp1 is required for formation of a CBF3–centromere complex [19].

There are several potential explanations for Skp1's multiple roles in G1 progression and kinetochore function. One is that Skp1 is primarily involved in kinetochore function, and *skp1* deficiencies induce a checkpoint control that causes a G1 block, stabilizing Sic1. A more interesting possibility is that Skp1 plays multiple roles: at the kinetochore through binding CBF3, at an undefined replication structure by binding Skp2 and the cyclin A–Cdk2 complex, and in the destruction of Sic1. Skp1's role in promoting protein destruction may be to recruit ubiquitination enzymes or the proteasome to specific targets. Bai *et al.* [17] suggested Skp1 might recruit the APC, an E3, to the kinetochore to promote destruction of proteins that maintain the cohesion of sister chromatids.

Skp1 may bind *via* F-box interactions to Cdc4, Skp2 and Grr1, which may in turn bind to substrate (Fig. 2). In addition to the many F-box-containing proteins, the diversity of Cdc53 homologs may provide combinatorial possibilities for recognizing specific substrates. Another possibility is that Skp1 and F-box proteins direct Cdks to their site of action. Skp1 might couple Cdks to the CBF3–kinetochore complex, to the site where Sic1 is degraded, or to the site where cyclin A–Cdk2 directs DNA replication. According to this model, Skp1 would promote phosphorylation, the subsequent binding of Cdc53 complexes or their homologs, and ultimately destruction.

The cullin family

The identification of a *CDC53* homologue, *cul-1*, in a screen for genes inhibiting hyperplasia in *Caenorhabditis elegans* is a sign of the general importance of these genes in cell-cycle control [20]. Kipreos *et al.* [20] found that *cul-1* (also called *lin-19*) is required for cells to exit the cell cycle, and that in its absence cell division occurs inappropriately. Although cell fate, differentiation and quiescence were apparently not affected in a variety of lineages, *cul1* mutants produced abnormally small cells and excessive cell divisions.

Cloning of *cul1* revealed the similarity of its product to Cdc53 [20]. Database searching with the *cul1* sequence revealed a family of at least six human, five nematode and three yeast genes, the products of which are now termed cullins. Furthermore, mutation in a gene homologous to *CDC4* also caused hyperplasia in *C. elegans*. Together, these results suggest that the Cdc34/Cdc4/Cdc53 pathway is highly conserved, but may integrate a variety of signals. These include nutritional signals in yeast, growth factors like TGF-β, and possibly extracellular signals such as cell-cell contact, cell attachment or contact inhibition.

The *cul1* results suggest that genes for components of the Cdc34 pathway could be tumor suppressor genes or oncogenes. While no ubiquitinating enzyme is known to be an oncogene product, the gene for a ubiquitin-deconjugating enzyme, *DOA4*, is homologous to an oncogene, *tre-2* [3]. Mutations that stabilize cyclins — either directly or by inactivating Cdk inhibitors or perturbing the ubiquitination machinery — may be of general importance in tumor progression. It may be profitable to search for mutations among genes in these pathways in human tumors or to examine how biochemical regulators of ubiquitination might be used therapeutically to block tumor growth.

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